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# Reactivity in ELISA with DNA-loaded nucleosomes in patients with proliferative lupus nephritis

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Short title: Reactivity with DNA loaded nucleosomes in lupus nephritis

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Abbreviations:

ELISA: enzyme linked immunosorbent assay

LN: lupus nephritis

mAb: monoclonal antibody

NcX: dsDNA loaded nucleosomes

SLE: systemic lupus erythematosus

SLEDAI: systemic lupus erythematosus disease activity index

## ***ABSTRACT***

Autoantibodies against nucleosomes are considered a hallmark of systemic lupus erythematosus (SLE). We compared in patients with proliferative lupus nephritis the diagnostic usefulness of a dsDNA-loaded nucleosome ELISA (anti-dsDNA-NcX) with ELISAs in which dsDNA or nucleosomes alone were coated. Defined monoclonal anti-DNA, anti-histone and nucleosome-specific autoantibodies were used to evaluate the accessibility of nucleosomal epitopes in the anti-dsDNA-NcX ELISA. Autoantibody levels were measured in these 3 ELISAs in 100 patients with proliferative lupus nephritis (LN) before immunosuppressive treatment. Inter-assay comparisons and associations with clinical and serological parameters were analysed. The panel of monoclonal antibodies revealed that all epitopes were equally accessible in the anti-dsDNA-NcX ELISA as in the two other ELISAs. Patients with proliferative lupus nephritis were positive with dsDNA-loaded nucleosomes in 86%, with DNA in 66% and with nucleosomes in 85%. In a non-lupus disease control group these frequencies were 1.6 % (2 out of 128) for both the anti-dsDNA-NcX and the anti-dsDNA ELISA and 0 % in the anti-nucleosome ELISA. The titers in the anti-dsDNA-NcX ELISA were high in a group of patients with LN that showed absent reactivity in the anti-DNA or low titers in the anti-nucleosome ELISA. Anti-dsDNA-NcX positivity was associated with higher SLEDAI scores within this group. Within nucleosome-based ELISAs, we propose the anti-dsDNA-NcX ELISA as the preferred test system.

***Key words:*** lupus, lupus nephritis, anti-nucleosome, anti-dsDNA, anti-DNA loaded nucleosome

## ***1. Introduction***

Autoantibodies against chromatin are considered a hallmark of the autoimmune disease systemic lupus erythematosus (SLE). Particularly, chromatin containing apoptosis-associated post-translational modifications are targeted (Dieker et al. 2007; van Bavel et al. 2009, 2010 and 2011). It is thought, that a disturbance in apoptosis or the removal of apoptotic cells results in persistence of circulating modified chromatin that is able to activate the immune system (Dieker et al. 2002; Fransen et al. 2009<sup>a</sup>; 2009<sup>b</sup>). This leads to the formation of anti-chromatin autoantibodies that form immune complexes with the circulating chromatin and can deposit in the glomerulus, thereby inciting a severe local inflammation (van der Vlag and Berden 2011). Nucleosomes are the basic units of chromatin and consist of double-stranded (ds)DNA wrapped around an octamer of histones. Nucleosomes are crucial as driving autoantigen in SLE and important initiators for glomerular lesions. Intrarenal perfusion of anti-DNA monoclonal antibodies complexed to nucleosomes led to an extensive binding to the glomerular basement membrane and proteinuria in contrast to non-complexed monoclonals. (Kramers et al. 1994). Elution studies from glomeruli of lupus mice revealed that anti-nucleosome antibodies were deposited first, later in time followed by anti-dsDNA antibodies (van Bruggen et al. 1996). In glomerular deposits in patients with proliferative LN, we could identify nucleosomes (van Bruggen et al. 1997). This finding was further corroborated by Rekvig and colleagues. With co-localization immunoelectron microscopy, nucleosomes were found to be co-localized with *in vivo* deposited IgG in both murine (Kalaaji et al. 2006) and human lupus nephritis (Kalaaji et al. 2007). In a joint endeavour with Professor Moh Daha, to whom this special issue of Molecular Immunology is devoted, we found that nucleosomes and C1q could bind *in vitro* to conditionally immortalized human glomerular endothelial cells. Bound nucleosomes served as targets for anti-nuclear antibodies from patients with SLE. This binding led to activation of the classical pathway of

complement. Anti-C1q antibodies amplified this complement activation (O'Flynn et al. 2011). Taken together these data indicate that both nucleosomes and dsDNA are important nephritogenic targets in LN.

Recently, a new ELISA was described that uses dsDNA coated onto nucleosomes as antigen (Biesen et al. 2011). At a specificity of 99% this ELISA (anti-dsDNA-NcX) shows a higher sensitivity (59.9%) compared to the anti-dsDNA (35.8% ) and anti-nucleosome ELISA (53.6%), as measured in 207 SLE patients, 357 disease controls (162 patients with rheumatoid arthritis, 88 patients with Sjögren's syndrome, 81 patients with systemic sclerosis and 26 patients with myositis) and 400 healthy donors.

Our objective for this study was to analyse the possible masking effect of dsDNA loading onto nucleosomes on the accessibility of nucleosomal epitopes, by using a panel of well-defined anti-dsDNA, anti-histone and nucleosome-specific monoclonal autoantibodies (mAb). In addition, we compared in patients with proliferative lupus nephritis the antibody reactivity in plasma using a coating of DNA-loaded nucleosomes (dsDNA-NcX), dsDNA or nucleosomes in ELISA and compared this to the reactivity in 128 non-lupus disease controls.

## **2. Methods**

### **2.1. Patients**

Plasmas were collected from patients with biopsy-proven proliferative lupus nephritis (at that time classified as WHO class III or IV (Churg et al. 1995) included in the first (Grootscholten et al. 2006; Arends et al. 2012) or second Dutch Lupus Nephritis Study (Arends et al. 2014). All patients fulfilled  $\geq 4$  American College of Rheumatology criteria for SLE. Samples at study entry were available from 100 patients. Disease activity was measured using the SLE disease activity index (SLEDAI) (Bombardier et al. 1992). Activity in the Farr assay and levels of complement C3 and C4 were measured routinely in the local diagnostic facility. The use of the patients' plasma was approved by the local ethics committee and by written consent from the patients. Patients (n = 128) referred for musculo-skeletal complaints to the Royal National Hospital for Rheumatic Diseases in Bath (UK) served as non-lupus disease controls. The blood sample was drawn at the first visit. Follow-up revealed various rheumatic diseases (rheumatoid or psoriatic arthritis, osteoarthritis, (fibro)myalgia, Raynaud's phenomenon, cutaneous sclerosis, various forms of vasculitis). Patients diagnosed with SLE during follow-up were excluded.

### **2.2. ELISAs**

The anti-dsDNA-NcX, anti-dsDNA and anti-nucleosome ELISA (Euroimmun Medizinische Labordiagnostika AG, Lübeck, Germany) were performed as published previously (Biesen et al 2011). Briefly, 100  $\mu$ l of patient samples (diluted 1:200 and 1:500) and positive and negative controls supplied by the manufacturer were transferred to pre-coated plates and incubated for 30 minutes at room temperature. A standard curve was generated from three calibration sera supplied by the manufacturer (10, 100 and 800 international units (IU) for the anti-dsDNA-NcX and anti-dsDNA ELISA; and 2, 20 and 200 relative units (RU) for the anti-

nucleosome ELISA). Subsequently, plates were washed, incubated with peroxidase-labelled anti-human IgG (30 minutes), washed again, and incubated (15 minutes) with substrate solution. The reaction was stopped with 0.5 M sulphuric acid and the absorbance was measured at 450 nm in a microplate reader (Biorad, Veenendaal, The Netherlands). IU and RU values were calculated using the respective standard curve. Cut-off values provided by the manufacturer were used (100 IU/ml for the anti-dsDNA-NcX and anti-dsDNA ELISA; and 20 RU/ml for the anti-nucleosome ELISA). The following mAb were tested in a dilution range: anti-dsDNA mAbs #36 and #42 (Kramers et al. 1994); anti-histone mAbs #34 (van der Heijden et al. 2005), KM-2 (Dieker et al. 2007) and BT164 (van Bavel et al. 2011); nucleosome-specific mAbs #32 (Kramers et al. 1994), BT95, BT116, BT124, BT131 and BT162 (van Bavel et al. 2010). For detection a peroxidase-labelled anti-mouse IgG(H+L) (Southern Biotechnology Associates, Birmingham, U.S.A.) was used.

### ***2.3. Statistical analysis***

Statistical analysis was performed using PASW Statistics 18 (SPSS, Chicago, Illinois, U.S.A.) and Graphpad Prism (Graphpad software, San Diego, California, U.S.A.). Correlations were studied using Spearman's rank correlation. Mann-Whitney U test was used to analyse differences between groups. A p value <0.05 was regarded as significant.



### **3. Results**

#### **3.1. Effect of dsDNA loading onto nucleosomes on the accessibility of nucleosomal epitopes**

Loading dsDNA onto coated nucleosomes in ELISA may lead to masking of epitopes both on dsDNA as well as on the nucleosome. Therefore, we tested the reactivity of a well-characterised panel of anti-dsDNA, anti-histone and nucleosome-specific monoclonal antibodies (mAbs). The effective concentration of the antibody giving 50% of the maximal signal (EC50) in the respective anti-dsDNA-NcX, anti-dsDNA and anti-nucleosome ELISA is depicted in Figure 1. Anti-DNA mAbs showed similar EC50 values in all three ELISA. Anti-histone and nucleosome-specific mAbs showed EC50 values in the anti-dsDNA-NcX ELISA similar to the anti-nucleosome ELISA, and were, as expected, negative in the anti-dsDNA ELISA.

#### **3.2. Reactivity in ELISA of patients with proliferative lupus nephritis**

We tested plasmas of a cohort of patients with proliferative lupus nephritis before immunosuppressive treatment in the anti-dsDNA-NcX, anti-dsDNA and anti-nucleosome ELISA (Figure 2a). Of the patients, 86% were positive in the anti-dsDNA-NcX, 85% in the anti-nucleosome ELISA, and 66% in the anti-dsDNA ELISA (Figure 2b). Twenty-one patients positive in the anti-dsDNA-NcX ELISA were negative in the anti-dsDNA ELISA, while 5 patients were negative in the anti-nucleosome ELISA. Only 5 patients being either positive in the anti-dsDNA (n=1) or the anti-nucleosome (n=4) ELISA were negative in the anti-dsDNA-NcX ELISA. No significant correlation of anti-dsDNA-NcX titers with the SLEDAI score was present. We made three groups on the basis of titers in the anti-dsDNA-NcX ELISA, <300 IU, between 300 and 900 IU and >900 IU (Figure 2c). Remarkably, in the 2 groups with anti-dsDNA-NcX titers between 300 and 900 IU, and > 900 IU, some patients showed much lower reactivity in the anti-dsDNA and/or anti-nucleosome ELISA. Correlation

of dsDNA-NcX and nucleosome reactivity showed two distinct populations (Figure 2d). The group of patients with a higher dsDNA-NcX reactivity compared to nucleosome reactivity showed a higher mean DNA reactivity ( $P < 0.0001$ ; Figure 2e), although some patients were negative in the anti-dsDNA ELISA. Higher anti-dsDNA-NcX titers were associated with higher SLEDAI scores ( $p = 0.045$ ; Figure 2f) and lower complement C4 levels ( $p = 0.036$ ), but not C3 ( $p = 0.083$ ) (both not shown). We found no significant difference between the two groups for clinical outcome parameters (Table 1) and no correlation with serum creatinine or proteinuria, before treatment or at last follow-up.

### ***3.3 Reactivity in ELISA of non-lupus disease controls***

In addition, we tested the reactivity of 128 non-lupus control patients, which is also depicted in Fig 2A. In both the anti-dsDNA-NcX ELISA and the anti-dsDNA ELISA, 2 of the 128 patients were positive, while none were positive in the anti-nucleosome ELISA. The 2 patients positive in both the anti-dsDNA and anti-dsDNA-NcX ELISA were identical and diagnosed as adult onset Still's disease and seronegative rheumatoid arthritis. Using this control group, we calculated the specificity to be respectively 98.4 % for the anti-dsDNA-NcX and the anti-dsDNA ELISA and 100 % for the anti-nucleosome ELISA.

## ***4. Discussion***

### ***4.1. Accessibility of nucleosomal epitopes***

We tested the anti-dsDNA-NcX ELISA for the accessibility of nucleosomal epitopes using defined anti-dsDNA, anti-histone and nucleosome specific mAbs. Their reactivity in the anti-dsDNA-NcX ELISA was comparable to the reactivity in the anti-dsDNA and/or anti-nucleosome ELISA. This indicates that the loading of extra dsDNA onto nucleosomes in the anti-dsDNA-NcX ELISA does not alter the accessibility of the various nucleosomal epitopes. Therefore, it seems that the anti-dsDNA-NcX ELISA system can be used to test all specificities of anti-nucleosome c.q. anti-chromatine antibodies (i.e. anti-dsDNA, anti-histone and nucleosome-specific antibodies).

### ***4.2. Comparison of ELISAs***

In patients with proliferative lupus nephritis the prevalence of positivity in the anti-dsDNA-NcX and anti-nucleosome ELISA was equal (86% and 85% respectively), but higher than in the anti-dsDNA ELISA (66%). Twenty-three patients showed significantly higher titers in the anti-dsDNA-NcX ELISA compared to the anti-nucleosome ELISA. These patients showed also a higher mean titer in the anti-dsDNA ELISA. This suggests that the anti-dsDNA-NcX ELISA provides a better antigenic platform for anti-dsDNA autoantibodies than the anti-nucleosome ELISA. The clinical relevance of a higher reactivity in the anti-dsDNA-NcX ELISA was indicated by a significantly higher SLEDAI score and lower C4 levels. Also reactivity in the Farr assay tended to be higher ( $p=0.1$ ). In addition, twenty-one patients positive in the anti-dsDNA-NcX ELISA were negative in the anti-dsDNA ELISA, suggesting either the presence of nucleosome-specific and/or anti-histone autoantibodies. These autoantibodies, additionally detected by the coating of nucleosomes in the ELISA, are specifically associated with SLE (Suer et al. 2004, Biesen et al. 2011, van der Vlag and

Berden 2011). Positive reactivity in the anti-nucleosome ELISA with a negative result in the anti-dsDNA ELISA is more frequently seen in patients with lupus nephritis (van der Vlag and Berden 2011). Five patients were negative in the anti-dsDNA-NcX ELISA, but either positive in the anti-dsDNA or anti-nucleosome ELISA (fig 2B). Reviewing the clinical charts and the laboratory findings, we could not detect common characteristics among these patients. So, we cannot explain why these patients tested negative.

We conclude that in patients with proliferative LN testing with a nucleosome-based ELISA has a higher sensitivity than with the anti-dsDNA ELISA, but has equal specificity. This higher sensitivity and equal specificity, has also been reported for SLE patients without nephritis (reviewed in van der Vlag and Berden 2011). Furthermore, the anti-dsDNA-NcX ELISA reveals a group of patients that has low reactivity with nucleosomes and/or dsDNA alone, whereas those patients have a significant higher SLEDAI score. Within nucleosome-based ELISA's, we propose the anti-dsDNA-NcX ELISA as the preferred test system, since we can detect much higher titers if anti-DNA antibodies are present.

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### *Disclosure statement*

The ELISA assays were provided by Euroimmun. WS is a board member of Euroimmun AG.

The other authors declare that they have no financial or other conflict of interest.

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## LEGENDS

**Figure 1.** DNA loading of nucleosomes does not mask accessibility of anti-chromatin epitopes. Reactivity of a panel of anti-nucleosome mAb in the different ELISAs. Reactivity of defined mAbs was tested in the anti-dsDNA-NcX ELISA and compared with the anti-dsDNA and anti-nucleosome (Nucl) ELISAs. The effective concentration of the antibody giving 50% of the maximal signal (EC50) in the respective ELISA is depicted. The accessibility of tested nucleosomal epitopes is not hampered in the anti-dsDNA-NcX ELISA.

**Figure 2.** The anti-dsDNA-NcX ELISA reveals higher antibody titers. Plasma reactivity of SLE patients (n=100) at onset of proliferative lupus nephritis in anti-dsDNA, anti-nucleosome (Nucl) and anti-DNA-loaded nucleosomes (dsDNA-NcX) ELISAs. (a) Plasma samples from patients with proliferative lupus nephritis were tested in all three ELISAs. In addition, the reactivity of non-lupus disease controls (n=128) was tested. (b) Comparison of plasmas that tested positive in the anti-dsDNA-NcX ELISA, the anti-nucleosome and/or anti-dsDNA ELISA shown in a Venn diagram. (c) Patients samples were divided in 3 groups based on their reactivity in the Anti-dsDNA-NcX ELISA. (d) Correlation of anti-dsDNA-NcX and anti-nucleosome titers shows two populations of patients. (e) Patients with a higher titers in the anti-dsDNA-NcX ELISA compared to the anti-nucleosome ELISA show a higher mean titer in the anti-dsDNA ELISA. (f) Patients with higher titers in the anti-NcX ELISA have a higher SLEDAI score.

Table 1. Comparison of clinical and laboratory parameters for patients showing equal (dsDNA-NcX~Nucl) or higher (dsDNA-NcX>>Nucl) reactivity in the anti-dsDNA-NcX ELISA compared to the anti-nucleosome (Nucl) ELISA

	<b>dsDNA- NcX~Nucl</b>	<b>dsDNA- NcX&gt;&gt;Nucl</b>
Patients (n)	41	19
Age (years)	34.2 (17-59)	35.6 (19-56)
Female/male	36/5	18/1
Serum creatinine (μmol/l)	124 (58-472)	131 (67-236)
Proteinuria (g/24h)	4.4 (0.1-9.2)	3.4 (0.5-6.7)
Farr assay (U/ml)	304 (0-3000)	846 (13-6940)
Sustained doubling of serum creatinine (n, %)	3 (7%)	3 (16%)
Renal relapse (n, %)	8 (20%)	4 (21%)
Lupus nephritis as 1st manifestation (n, %)	16 (39%)	10 (53%)
Serum creatinine last follow-up (μmol/l)	99 (51-418)	124 (45-440)
Proteinuria last follow-up (g/24h)	0.48 (0-2.3)	0.38 (0-1.9)
Death (n, %)	6 (15%)	2 (11%)